Fracture healing and the underexposed role of extracellular vesicle-based crosstalk

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Fracture healing and the underexposed role of extracellular vesicle-based crosstalk

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Abstract

The process of fracture healing is complex and requires aninteraction of multiple organ systems. Cell-cell communication is known to be very important during this process. Extracellular vesicle (EVs) are small membranous vesicles generated from a variety of cells. Proteins, RNAs, small molecules and mitochondria DNA were found to be transported among cells through EVs. EV-based crosstalk represents a substantial cell-cell communication pattern, that can bothinteract with cells through molecular surfaces, and transfer molecules to cells. These interactions can assist in the synchronization of cellular functions among cells of the same kind, and coordinate the functions of different types of cells. After activation, platelets, neutrophils, macrophages, osteoblasts, osteoclasts, and mesenchymal stem cell (MSC) all secrete EVs, promoting the fracture healing process. Moreover, some studies have found evidence that EVsmay be used for diagnosis and treatment of delayed fracture healing, and may be significantly involved in the pathophysiology of fracture healing disturbances. In this review, we summarize recent findings on: 1) EVs released by fracture healing-related cells, and 2) EV-mediated communications during fracture healing. We also highlight the potential applications of EVs in fracture healing. Lastly, the prospect of EVs for research and clinical use is discussed

Keywords

Extracellular vesicle; Osteoblast; Osteoclast; Platelet; Endothelial cell; Intercellular communication; Crosstalk; MiRNA

Fracture healing is a complex process, involving the interaction of immunity, bone, and vessel systems. During bone healing, cells from different systems have to communicate with each other. There are three cell-cell signal transduction modes thatare commonly known and accepted(1):

1) secretion molecules,

2) cell-cell contact, and

3) tunnelingnanotubules.

Recent studies have introduced the potential for communication viaextracellular vesicles (EVs)(2, 3).It is knownthatEVs from osteoblasts, chondroblasts, and osteoclasts are important for tissue calcification and bone remodeling. Furthermore, studies have shown a crucial role of EV-based crosstalk in the physiological and pathophysiological processes of coagulation, inflammation, and osteogenesis(3, 4). These processes are involved in fracture healing, and thus,EV-based crosstalk communication occurs throughout the whole process of fracture healing. As different types of EVs can be released under different conditions(5, 6), this review focuses on studies dealing with the role of EVs in the process of fracture healing.

1. What areEVs

EVs are membranous vesicles generated from a variety of cells, with diameters ranging from 30-5000nm. Three kinds of extracellular EVshave been observed: exosomes, shedding microvesicles, and apoptoticblebs(7). Exosomes are cup-shaped membranous EVs, with a diameter of 30-100 nm.They generated by inward budding of endosomal membranes into large multivesicular bodies (MVBs), and secreted by exocytosis(8), with surface markers like Alix, HCS70, CD63, CD81, and CD9 (7). Shedding microvesicles are generated by blebbing and shedding of the plasma membrane from almost all cell types, with a diameter of 100-1000 nm, and have cell markers such as selectins and integrins(9). In some publications, shedding microvesicles were also called microparticles(10), while in some others, microparticles referred as those isolated from platelets or endothelial cells(9). Apoptoticblebs are membrane vesicles generated by apoptotic or dying cells(7), with a diameter of 50-5000 nm. Inside

of these EVs, proteins, RNAs, small molecules and mitochondria DNA can be found. In this article, we focus on exosomesand shedding microvesicles.

2. EV-based crosstalk

EVs are competent information mediators. The membranous structure of EVsenables them to transport moleculeswithin their inner cytosol, and membrane-dependent molecules at the surface. The specialties of EV-based molecular transport are:

1) Transport of RNAs between cells,

2) Transport of components of specific pathways

3) Transport of surface molecules and membranous molecules.

The transportation of these molecules enables them to coordinate cellular reactions;this process can control gene expression by regulating mRNA turnover, ultimately contributing to the epigenetic and proteomic properties of the target cells(11),as well as changing the maturation and differentiation of target cells(12). At the same time, cells can activate surface molecular pathways of remote cells.

Organelles have also been found to be transferred between different cells by EVs(13). Studies have found mitochondrial DNAs in EVs from platelets, astrocytes, glioblastoma, and bone marrow-derivedstromal cells (14, 15).According to the results from recent studies, the release of mitochondria is able to elevate inflammatory reactions (14, 16).

In addition to other mechanisms, the interaction of EVs with cells includes 1)surface molecules, 2) EV-cell fusion, and 3) cargo releasing.

EVs carry surface molecules and transmembrane proteins on their surfaces and, therefore, include characteristics of the cells they were released from. These surface proteins help to interact with specific target cells, increase the specificity of cargo deliveryand trigger signaling cascades via receptor interactions(7).

Through EV-cell fusion, proteins and RNAs can be transported into recipient cells. The EV-cell interactions and fusions are mediated by receptor binding (17). Using membrane-bound structures, EVs can receive signals and fuse with their target cells(3). Due to the presence of surface receptors, EVs only interact with cells they recognize(18).

EVs also have the potential to reach the extracellular spaces and blood, releasing their content and membrane components(18, 19).EVs contain proteinase, cytokines, growth factors, and organelles(1, 20) that function in the extracellular matrix.

3. EVsfromfracturehealing-related cells

3.1 OsteoblasticEVs (OBEVs)

Osteoblasts play a central role in the fracture healing process. It has already been reported that osteoblasts directly communicate with osteoclasts (4, 21), mesenchymal stem cell (MSC)(22, 23), and others (24),via EVs. Proteomic analysis of OBEVs/exosomesrevealed (25)proteins that modulate osteogenesis,which are involved in pathways such as the eukaryotic initiation factor 2 (EIF2) signaling pathway, integrin signaling pathway, Rho-guanosine triphosphate (Rho-GTP) signaling pathway, and mammalian target of rapamycin (mTOR) signaling pathway(25).

OBEVswere observed to induce differentiation of multi-potential stem cells into osteoblasts (24, 26, 27). OBEVs/exosomes from mineralizing osteoblasts have the ability to promote the differentiation of bone marrow stromal cellsinto osteoblasts, a process which is mediated by theWnt signaling pathway(27). Several studies have also found evidence to show that osteoblastscommunicate with osteoclasts through OBEVs. Receptor activator of nuclear factor-κB ligand (RANKL) proteins, which

are able to induce generation of osteoclasts, were found inEVs/microvesicles originated from osteoblasts(4).In rats, hypoxia stimulates therelease of EVs/microvesiclesfrom osteoblasts,containing vesicular adenosine triphosphate (ATP), which was proved to influence functions of osteoclasts(28).

3.2 Mesenchymal stem cell(MSC)-derived EVs (MSCEV)

MSCs play an active role in the processes of immune modulation and tissue regeneration. Cytokines, micro-RNAs (miRNAs), surface molecules, and signaling pathway components from MSCEVshave been shown to be involved in cell-cell communications.

Ischemia-activated MSCs can release EVs with high concentrations of platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and other angiogenesis-related molecules(29). The release of these factors into theextracellular matrix promotes angiogenesis and osteogenesis.

Important fracture healing-related pathways have been found in Evs/microvesiclesfrom activated MSCs, including in the Wntsignaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, transforming growth factor (TGF) signaling pathway, peroxisome proliferator-activated receptors (PPAR) signaling pathway, and the bone morphogenetic protein (BMP) signaling pathway(30). A variety of surface molecules from MSCEVs/microvesicles were found to be involved in fracture healing processes, including PDGF receptors, EGF receptors, ezrin (EZR), integrins, and IQ motif containing GTPase activating protein 1(IQGAP-1) (30). These pathways and molecules are involved in the recruitment and differentiation of MSCs(31),the regulation of endothelial cell proliferation, angiogenesis(32),as well as epithelial proliferation(33).

A variety of miRNAshave been detected inMSCEVs/microvesicles(34),some of which were also shown to have functions related to fracture healing. Nakamura et al. (35)reported that MSC-derived miRNA promotes myogenesis and angiogenesis. MiR-196a(22)was observed to promote osteogenesis, while miR-378(36), miR-223(37), and miR-100(38) were found to inhibit osteogenesis. MiR-378(39)and miR-223(40) were also found to be involved in angiogeneticremodeling.It is also reported

that, at different time points during fracture healing, MSCs release EVswith different profiles of miRNAs, which areinvolved in osteoblastogenesisorleukocyte migration (23)

3.3 Endothelial EVs (EEVs)

The generation of endothelial EVswas found tobe stimulated by inflammatory cytokines,bacterial lipopolysaccharides, reactive oxygen species, thrombin, C-reactive protein, and uremic toxins(41).The generation of EEVsafter TNF-αand thrombin stimulation involvesseveral signaling pathways including thecaspase, rho-kinase, nuclear factor kappa B (NF-κB) pathways, andMAPK(41, 42). Tumor Necrosis Factor α(TNF-α), interleukin -1 (IL-1), and intergrin-1amay amplify the release of EEVsthroughthe NF-kβ and MAPK pathways(43).

EEVshave been found to be important elements in the processes of coagulation, inflammation, angiogenesis, and osteogenesis. In the early phase of fracture healing, phospholipids and proteins at the surface of EEVswere found to control the coagulation equilibrium, among them are endothelial protein C receptor (EPCR), thrombomodulin (TM),tissue factor (TF), von Willebrand factor (vWf), tissue plasminogen activator (tPA), and Phosphatidylserines (PS)(43-46).Inflammation-activated EEVs bear inflammation modulating moleculessuch as platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM-1),and endothelial-selectin (E-selectin)(42, 47). These molecules are known to have diverse roles in vascular biology, including angiogenesis, thrombosis, and regulation of multiple stages of leukocyte migration.At the same time, studies have reported thatEEVs modulate proliferation, angiogenesis, and apoptosis of endothelial cells through RNA transportation. Horizontal transportation of miRNAsbetween endothelial cells byEEVs/microvesicles promotes angiogenesis following Akt activation and endothelial nitric oxide synthase (eNOS) expression(48), and promotes vascular endothelial reparation(49).Furthermore, EEVs contain matrix metalloproteases, which are involved in the extracellular matrix degradation, as well as the release of growth factors that play crucial roles inbone remodeling and angiogenesis(50, 51).The role of EEVs in osteogenesis has beenreported in several studies. Inflammation-induced EEVexpress molecules,such asPECAM-1 and ICAM-1,which were found to promote osteoclastogenesis(52, 53). EEVs/microvesicles from TNF-α-stimulated endothelial cells were also found to containa significant amount of BMP-2, and were able to enhance vascular smooth muscle cell osteogenesis and calcification(54).

3.4 OsteoclasticEVs (OCEVs)

Osteoclasts are an important element in fracture healing and bone remodeling. A study tested the profile of OCEV-miRNAs from TNF-α and RANKL-stimulated osteoclasts, showing a variety of miRNAs (miR-378, miR-223, mir-210, miR-21, miR-155, miR-146a, and miR-199a) that may be involved in angiogenesis, osteogenesis, and chondrogenesis(55). RANK was also found to be present on the surface of osteoclasticEVs, and is capable of regulatingosteoclastogenesis(56). More studies are needed to investigate the communication functions of OCEVs.

3.5 Macrophage and monocyte-derivedEVs

Macrophages and monocytesare important for both initiation ofinflammatory reactions and tissue repair. Granulocyte-macrophage colony stimulating factor (GM-CSF)(57), Lipopolysaccharides (LPS)(58), and Fas-Ligend(59)were found to be able tostimulategeneration of EVs by macrophages.

EVs/microvesicles from cultured macrophages have been shown to transport functional RNA molecules into target cells, including monocytes, endothelial cells, epithelial cells, and fibroblasts(57). EVs/microvesicles from monocytes and macrophages were found tobe rich of tissue factor (TF), which can bind platelets through P-selectin glycoprotein ligand-1 (PSGL-1) on the EVs, and P-selectin on the platelets; thereby enhancing coagulation(60).EVs/microvesicles from monocytes were observed to interact with endothelial cells, inducing pro-coagulatory and pro-inflammatoryendothelial phenotypes(59). *In vitro* capillary tube formation and *in vivo* angiogenesis assays have shown that monocyte-derivedEVs/microvesicles have strong pro-angiogenic activities(61).MSCs/exosomes incubated by monocyte-derivedEVs showed increased runt-related transcription factor 2 (RUNX2) and BMP-2, indicating the osteogenic differentiation of MSCs(58).

3.6 Neutrophil-derivedEVs (NEVs)

The initiation of an inflammatory and coagulatory response has the potential to stimulate the generation of NEVs. TNF-α, the complement system, IL-8, platelet activating factor protein kinase C activator, and nitric oxide synthase inhibitor have been found to increase the production of NEVs(62).It has been reported that, under inflammatory conditions, the amount of NEVs will increase significantly, compared to normal conditions(63, 64).

Although there are no studies referring to the functions of NEVs in fracture healing, several studies have indicated a role of NEVs in fracture healing-related reactions. During coagulation, platelets were found to be activated by NEVs in an Akt phosphorylation-dependent manner(65). In addition, NEVsappear to be important inflammation modulators, as they may have both anti-inflammatory(10, 66)and pro-inflammatory functions(10, 67, 68), depending on the background of the microenvironment(10, 62). Studies on proteomes of NEVs/microvesicleshave revealed over 400 proteins, covering mainly integrinsignaling, EGF, and FGF signaling pathways(10). Although it has not beenprovenyet, the EGF and FGF signaling pathways found in NEVs/microvesicles may stimulate the differentiation of MSCs and angiogenesis(10).

3.7 Platelet-derivedEVs (PEVs)

Platelets are activated under coagulatory and inflammatory conditions, with the subsequent release of large amounts of functional PEVs(69). A study demonstrated that 87% of the release of platelet-activating factor (PAF), a key factor for triggering inflammatory and thrombotic cascades, was associated with EVs(70, 71). There is also evidence that PEVs may transfer platelet-specific immunoreactive antigens to the surface of other cells, modulating cellular functions(72).

In the early phase of fracture healing, expansion of coagulation platelet-platelet crosstalk is one of the most important featuresof PEVs. PEVs express anionic phospholipids (AP) and TF on their surfaces;arachidonic acids (AA) from platelets were reported to have the potential to activate the clotting cascades in platelets(73, 74). PEVshave also been found to be able to bind to the subendothelial matrix *in vitro* and *in vivo*, acting as substrates for further platelet binding(75).

Posttraumatic modulation of inflammation by PEV-based crosstalk was shown in several publications(76). In a clinical study, high posttraumatic levels of PEVswere associated with the development of acute respiratory distress syndrome (ARDS) and multiple organ failure(MOF)(73). Through PEVAA and bioactive prostanoids, PEVswere found to promote inflammatory reactions by activating MAPK pathways(77). Through the MAPK pathway, PEVs can induce the production of

important inflammation mediators like Cyclooxygenase-2 (COX-2) and prostacyclin (PGI2), in human endothelial cells(77). PEVs were shown to activate monocytes and macrophages, and reprogram their functions towards a phagocytic phenotype, by regulating their RNA expression(78) and activating theNF-κβ pathway(79). Neutrophils(80)and leukocytes(16, 81) are also the targets of PEVs. One study found that the binding of PEVs to neutrophils can induce a significant increase in phagocytic activity in aconcentration-dependent manner(80).Mitochondria found in PEVs can perform as inflammation mediators, activating leukocytes and neutrophils, causing inflammatory responses(16).

Studies haveshown that PEVs can modulate the proliferation, survival, chemotaxis, and function of endothelial cells through the VEGF pathway(82) and by argonaute 2 (Ago2)-microRNA complex-delivering(83). Ithasalso been demonstrated that PEVs are able to stimulate mitogenic activity of bone cells, contributing to bone regeneration(84).In addition, a study showed that surface molecules on PEVscan activehematopoietic cells by chemotaxis, increasing adhesion, proliferation, and survival, and activating signaling cascades including MAPK p42/44, phosphatidylinositol-3 kinase (PI-3K),AKT, and Signal transducer and activator of transcription (STAT) proteins(85).

3.8 Other cellular EVs involved in fracture healing processes

Other cell types have also been observed to releaseEVs under inflammatory conditions. Even though there is limited evidence in the literature, the existing data may provide the first evidence for the role of EV-based crosstalk in fracture healing. Erythrocytes activated by lysophosphatidic acid can secrete pro-coagulatoryEVs, which contribute to coagulation processes(86). Vascular smooth muscle cells incubated by LDL are found to produce TF-bearing EVs, promoting coagulation cascades(87).

EVs from chondrocytes have been detected in the growth plate. EVs are released in a polarized fashion from the lateral edges of growth plate chondrocytes. They have been found to be involved in the mineralization of bone matrix(88). Later, BMP, VEGF, bone sialoprotein (BSP), osteonectin, and osteocalcin werealso found in these EVs, highlighting the communicative role of chondroblasticEVs(88-91). VEGF, if released into the matrix, regulates angiogenesis and osteogenesis, and stimulates endothelial cells, chondroblasts, and osteoblasts(92). BSPin EVs was shown to promote differentiation of osteoblasts and repair of bone defects(91), as well as to promote differentiation of

chondrocytes and osteoblasts(in growth periods) (90). Further, osteopontin, osteonectin, and osteocalcin were found to be able to inhibit mineralization *in vitro*(88).

4. EV-based crosstalk network during fracture healing

Fracture healing involves very complex systemic reactions. It involves sophisticated cooperation betweena variety of cells, cytokines, and inflammatory factors (figure 3). In this chapter, we will briefly overview the network of EV-based crosstalk during fracture healing, highlighting the networks of cytokines and inflammatory factors.

Coagulation and inflammatory reactions are the first steps for fracture healing. In this phase, the ruptured blood vessels, tissue necrosis, and other ongoing damage, stimulate the inflammatory cascades. The activation of platelets, neutrophils, and macrophages by hypoxia, coagulation, and inflammation are observed to produce, not only a variety of cytokines and inflammatory factors, but also large amounts of active EVs(1, 93). The appearance of EVs may indicate that they have important functions.

A short time after blood vessel and tissue injury, pro-coagulatoryEVs can be released from platelets and neutrophils. The positive feedback model of PEVsappears to further stimulate PEV productions (74), as well as activate and attract neutrophils(16, 80) and macrophages(78, 79). EEVs from activated endothelial cells also promote coagulation via EPCR, TM, TF, vWf, tPA, PS, and plasminogen(43, 94, 95).

Inflammatory background is important for fracture healing. EVs derived from a variety of cellsparticipate in the inflammatory regulationof the fracture site. PEVsare able to stimulate the differentiation of monocytes and macrophages, inducing the production of pro-inflammatory factors(78, 79). The activation of neutrophils results in secretion of NEVs, that also regulate inflammatory reactions.In addition, NEVs can increase the inflammatory response by the activation and

aggregation of macrophages(67), inducing production of IL-6, IL-8, MAC-1, and oxygen active species(10, 96). However, NEVs can also inhibit the aggregation of neutrophils (97), induce the production of anti-inflammatory factors from macrophages such as IL-10 and TGF-β1(66, 98) and inhibit pro-inflammatory actions of endothelial cells(10). Although EVs from MSCs have been shown to have the potential to modulate the inflammatory response, further studies are needed to clarify their role in the process of fracture healing.

A variety of EVs take part in angiogenesis modulation during fracture healing. EVs from platelets, neutrophils, macrophages, MSCs, and endothelial cells have been shown to have the ability to stimulate differentiation of stem cells or endothelial cells. EVs from MSCs(29, 31) and chondrocytes(92)have been shown to release multiple growth factors, providing a microenvironment for angiogenesis and tissue regeneration. Surface molecules from MSCEVs(32, 33)and EEVs(99)have also been shown to modulate angiogenesis. Pathway molecules and miRNAs from PEVs(83), MSCEVs(35), EEVs(48, 49, 100), and osteoclast-(101) and monocyte-derivedEVs(61)also participate in the modulation of angiogenesis.

During tissue regeneration and bone remodeling, multiple EVstake part in the process of activation of fibroblasts, chondroblasts, and osteoblasts. EVsfrom MSCshavea significant role in wound healing. They were found to enhance wound healing via promotion of collagen synthesis and angiogenesis(102). One study also found that MSC-derived exosomes causedan increase in proliferation and migration of fibroblasts(103). In the microenvironment of osteogenesis, a mixture ofEVshas been found to modulate the osteogenesis reactions. Matrix vesicles found in the growth plate serve as carriers of morphogenetic information to nearby chondrocytes and osteoblasts. These matrix vesicles were observed to be released from the lateral edges of growth plate chondrocytes, the osteoid-facing surfaces of osteoblasts, and the apical surfaces of odontoblasts(88). Cultured chondrocytes treated with these matrix vesicles showed a two- to three-fold increase in alkaline phosphatase activity (88). OBEVs contain miRNAs and pathway molecules, which have the potential to stimulate osteogenesis of MSCs(26, 27), and communicate with osteoclasts for bone remodeling(4, 28). Molecules from EEVs, such as BMP-2(54) and PECAM-1(104)have been shown to involve in the modulation of angiogenesis, osteogenesis, and bone remodeling.

In addition to their communicative functions, EVs from osteoblasts, chondroblasts, and osteoclasts are also involved in osteogenesis and bone remodeling. They are the practical workers for bone matrix generation and calcification. Aspects of matrix generation, calcification, and bone remodeling are reviewed elsewhere(105).

A summary of EV-based miRNAs related to osteogenesis, obtained from ExoCarta (12/2016)(106), can be found in Figure 1, Figure2, and Table 1.

5. The potential application value of EVs during fracture healing

5.1 Molecular diagnosis

Multiple components of EVs, especially miRNAs, are suggested as biomarkers for molecular diagnosis. Several studies have discussed the diagnostic value of EVs in neurological disorders(107), cardiovascular diseases(108), osteoporosis(48)and sepsis(109).

With regards to the skeleton, the diagnostic potentials have also been investigated. One study tested the proportion of EVs in rheumatoid arthritis (RA) patients. The authors found an increase of plasmatic CD3(+), CD14(+), CD19(+), CD41(+), CD62E(+) MPs, and urinary CD14(+), CD3(+), and CD19(+) MPs in RA patients with high disease activity(110). More interestingly, compared to a healthy population, the level of miR-92a was found to decrease 24 hours after a bone fracture, while it was decreased 14 days after a lumbar compression fracture(111). These studies showed the potential of EVs as a diagnostic tool. However, more research is needed to bring this marker into clinical practice as a routine parameter.

5.2 EV-based therapies

Comparedto proteins and RNAs, EV structures may be more tolerant to the microenvironment *in vivo*(112). Thus, the use of EEV-mediated delivery is expected to be more efficient than using soluble

proteins or RNA molecules. One study also indicated thatEV structures may be at lower risk of immunogenicity and toxicity(112).

The therapeutic potential of EVshas been studied in the treatment of several diseases, such as acute lung injury(113), myocardial ischemia/reperfusion injury (114), kidney injury (115), peri-implant bone defect(116) and others;the results appear to be promising. Moreover, some researchers have developed manufactured EVs to improve treatment of fracture healing. In this context, researchers aimed to remodel cell-derived EVs, thereby making them more effective for treatment. These small molecules, miRNAs, and exogenous proteins have been successfully delivered by EVs(117, 118).In addition, specifically generated gelatin EVs have been used as vehicles to transport molecules. With this technique, bone defects have been treated successfully by transporting BMPs, VEGFs, TGF-β, and other molecules(119, 120), to target cells. Poly(lactic-co-glycolic acid) (PLGA) nanoparticleswere also shown to be a potential vehicle in guiding bone regeneration(121).

6. Prospect for research and clinical use

To date, the mechanism of EVs involvement in bone matrix generation and mineralization has been thoroughly investigated. However, there is still little information about the role of information transfer during the process of fracture healing. Therefore, further studies should focus on this area of research. To date, knowledge gained from studies has only shown the important role of EV-based communication in fracture healing, while the more detailed mechanisms remain unknown.

Many potential advantages of EVs make them worthy for future studies. EVs can be easily collected from blood or body fluids, with comparable characteristics of their original solid cells; this is advantageous forthe diagnostic application of EVs. MiRNAs and surface molecules in EVsare more stable. However, little is known about the specificity of EVs in fracture healing-related states. Further studies are needed to look into the components and surface markers of EVs which may be able to predict the fracture healing states, and the potential threats.

The application ofEVs in tissue engineering offers many possibilities for treatment options. Researchers have tried to use the advantages of EVs in improving fracture healing and treating bone defects, with optimistic results. However, some restrictions limit theirlarge scale clinical use. For example, the production of EVs should be generated with more specific components, and needs to be produced in large scale. Thus, specific tissue-engineering cells maybe a good option for the next research step. Tissue-engineering cells or bacteria can produce highly concentrated and purified EVs. At the same time, highly specified surface molecules can accurately aim at targeted cells. In addition, the clearance of EVs*in vivo* also needs to be considered; more stable EVs may need to be developed for effective treatments. Furthermore, man-made EVsrepresent a very promising field for research and treatment.

7. Conclusion

EVs play an important role during fracture healing, with theirinformation transportation andbone matrix generation. While the mechanism ofEVsinvolvement in bone matrix generation is clear, the mechanism of EVs role in information transportation remains underexposed and is a field of research which requires further study. It is likely that, in the near future, EVs will be useful in clinical diagnosis and treatment.

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Figure 1. MV-originated miRNAs related to osteoblastic functions.
Runt-related transcription factor 2 (RUNX2), transforming growth factor-β(TGF-β), bone morphogenetic protein (BMP), phosphatidylinositol-3 kinase (PI3K), nuclear factor kappa B (NF-κB), histone deacetylases (HDAC), mitogen-activated protein kinase (MAPK), mechanistic target of rapamycin (mTOR)

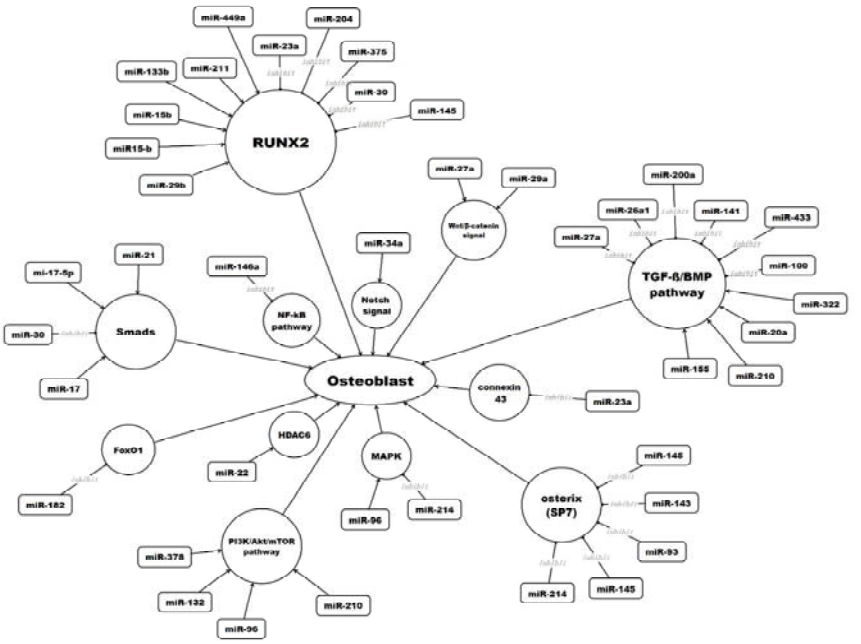

Figure 2. MV-originated miRNAs related to endothelialcell functions.
Runt-related transcription factor 2 (RUNX2), transforming growth factor(TGF), bone morphogenetic protein (BMP), phosphatidylinositol-3 kinase (PI3K), nuclear factor kappa B (NF-κB), histone deacetylases (HDAC), mitogen-activated protein kinase (MAPK)

extracellular signal–regulated kinases (ERK), mechanistic target of rapamycin (mTOR), Insulin-like growth factor 1 (IGF-1) platelet-derived growth factor receptor (PDGFR) Semaphorin-6A (SEMA6A) Phosphatase and tensin homolog (PTEN) Hypoxia-inducible factor 1-alpha (HIF-1α) vascular endothelial growth factor (VEGF)

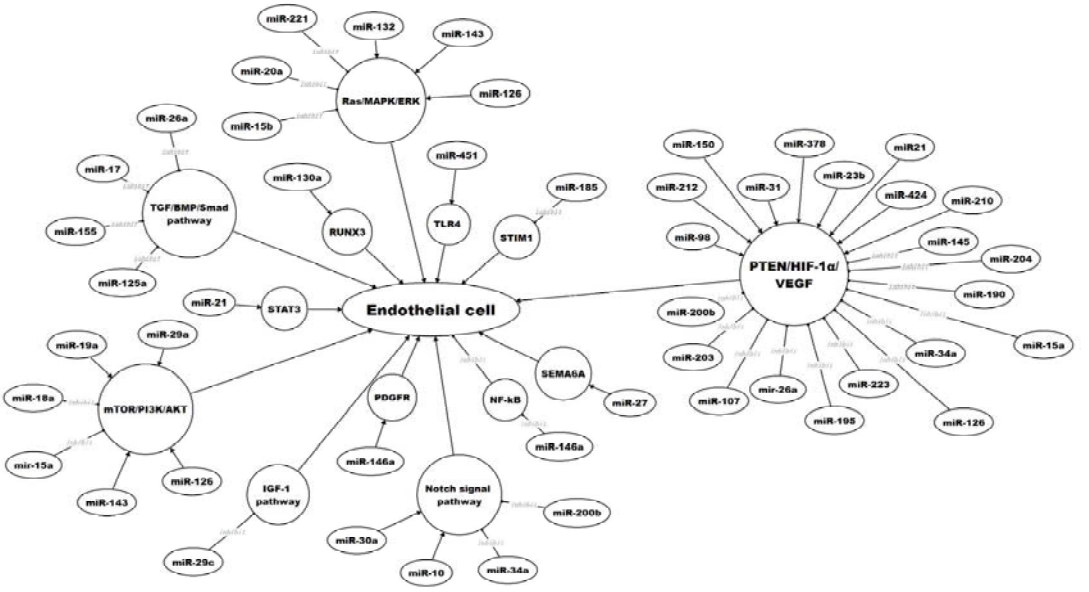

Figure 3. Known MV-based crosstalk mechanisms during fracture healing.

Abbreviations: anionic phospholipid (AP), tissue factor (TF), arachidonic acid (AA), platelet-activating factor (PAF), P-selectin glycoprotein ligand-1 (PSGL-1), platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI-3K), Signal transducer and activator of transcription (STAT), argonaute 2 (Ago2), endothelial protein C receptor (EPCR), thrombomodulin (TM), von Willebrand factor (vWf), plasminogen activator (PA), phosphatidylserines (PS), platelet endothelial cell adhesion molecule (PECAM), intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), bone morphogenetic protein (BMP), ezrin (EZR), IQ motif containing GTPase activating protein 1 (IQGAP-1), insulin-like growth factor-1 receptor (IGF-1R), stromal cell-derived factor 1 (SDF1), growth factor (GF), receptor activator of nuclear factor kappa-B ligand (RANKL)

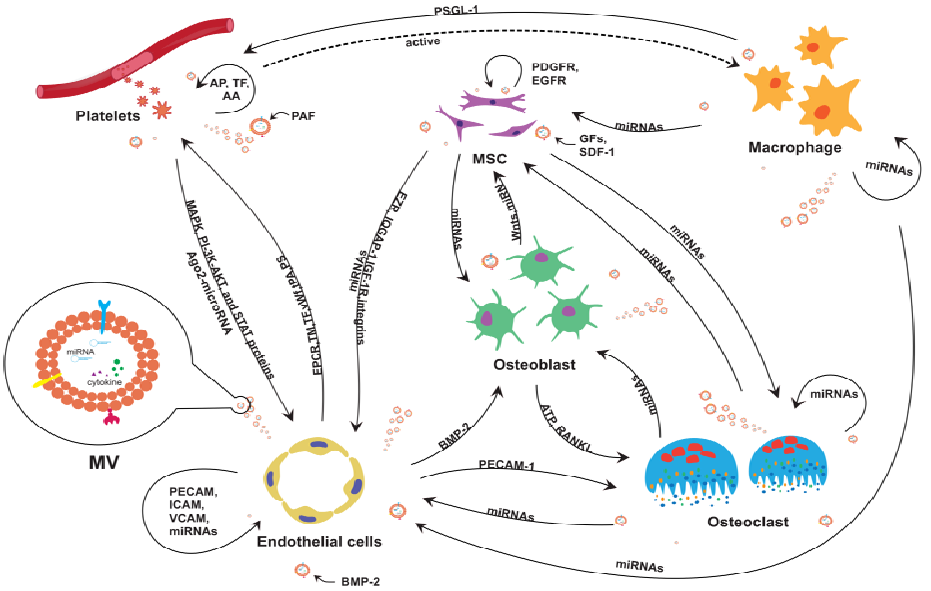

Table 1. summary of EV-based miRNAs related to osteogenesis

miRNA type	**Recipient cell**	**Donor cells**	**References**
miR-100	MSC	MSC	(122), (34), (38)
miR-125a	endothelial cell, osteoclast	MSC	(34), (123), (124)
miR-125b	osteoblast	MSC	(122), (34), (125),
miR-126	endothelial cell, MSC, osteoblast	endothelial cell	(49), (126), (127)
miR-133a	osteoblast	MSC	(34), (122)
miR-135a	osteoblast	MSC	(34), (122)
miR-137	osteoblast	MSC	(34), (122)
miR-150	endothelial cell	monocyte	(61)
miR-155	endothelial cell	osteoclast, monocyte	(55), (128)
miR-204	MSC	MSC	(34), (129)
miR-205	osteoblast	MSC	(34)
miR-21	osteoblast, chondrocyte, endothelial cell,	endothelial, osteoclast	(130), (55), (122), (131)

	MSC, osteoclast		
miR-210	osteoblast, chondrocyte, endothelial cell, MSC	osteoclast	(131), (132), (122), (133)
miR-217	osteoblast	MSC	(34), (122)
miR-221	endothelial cell	MSC	(34), (134)
miR-223	osteoblast,osteoclast, endothelial cell	MSC, osteoclast, macrophage	(34), (122), (135), (130), (55), (131)
miR-23a	osteoblast, MSC	MSC	(34), (122)
miR-24	osteoblast	MSC	(34), (122)
miR-30	osteoblast	MSC	(34), (122)
miR-31	endothelial cell, osteoclast, osteoblast	MSC	(34), (122)
miR-338	osteoblast	MSC	(34), (122)
miR-34	osteoblast	MSC	(34), (122)
miR-378	osteoclast, endothelial cell	osteoclast	(36, 55, 130),
miR-451	endothelial cell	MSC	(34)